IDENTIFICATION OF MUSHROOM MYCELIA USING DNA TECHNIQUES

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ABSTRACT

The suitability of using DNA techniques in the determination of relatedness of mushroom fruiting bodies to isolated mycelia was examined. Nine isolates of edible mushroom mycelia of genera Oudemansiella, Coprinus *and* Pleurotus *were identified using fruiting bodies as references. Polymerase Chain Reaction (PCR) in combination with Restriction Fragment Length Polymorphism (RLFP) analyses were carried out on fruiting bodies and mycelia of the isolates. The internally transcribed spacer region (ITS) of ribosomal RNA gene (rDNA) was amplified using ITS1 and ITS4 primers. The RLFP analysis was carried out on the regions amplified by PCR from fruiting bodies and mycelia of the isolates using three restriction enzymes:* MboI, HaeIII *and* HinfI. *Relatedness of the fruiting bodies and the mycelia was established by looking at DNA fragment band sizes and patterns. Banding patterns and fragment sizes of DNA obtained from mycelia and their corresponding fruiting bodies were identical and characteristic for the species. Using this technique, it was possible to sort out a case of mistaken identity of* Oudemansiella *fruiting bodies, which were interchanged with another mushroom specimen during packing. The method is fast, accurate, and could be used for routine screening of edible mushrooms of Tanzania for taxonomical purposes. For the latter purpose, it is required that the RFLP database of taxonomically known species is in place.*

INTRODUCTION

Mushrooms are fruiting stages of microscopic fungi of the classes Basidiomycetes and Ascomycetes. Since earliest times, edible mushrooms have been regarded to be a uniquely special food, a delicacy, health food, 'Foòd for the Gods' which was served only on festive occasions and to be medicine (Chang & Quimio 1989). At present mushrooms are considered to be food of high quality with a pleasant flavour, appealing texture and high nutritional value. More than 2,000 edible fungi are widely accepted for human consumption (Chang & Quimio 1989), although only a few are commercially cultivated. In Tanzania, more than 50 edible mushroom species have been identified (Eyssartier *et al.* 1999, Härkönen *et al.* 1995) and only one (*Pleurotus flabellatus*) is in cultivation at small scale.

Many Tanzanian tribes include mushrooms in their diets (Härkönen *et al.* 1995). Mushroom collection from the wild is done during the rainy season. In order to improve on the availability of mushrooms as a source of proteins and vitamins regionally, this agro-cultivation has to be promoted. Since many commercial strains are adapted to temperate climate, indigenous strains and species should be used for local cultivation. This will require a systematic study of the taxonomy and growth conditions of indigenous mushroom species and the substrates for their growth. It is imperative to conduct field surveys, collect and isolate mycelia from fruiting bodies and properly identify the species. The collection, characterisation and conservation of wild types is critical to assure availability of breeding material for commerciallycultivable mushrooms.

In developing countries of the tropics, the rich mushroom diversity combined with the present cultivation technologies offer great potential for improving productivity of small-scale mushroom growing. One of the recommendations which came out of the recent International Meeting on the Conservation and Utilization of Genetic Resources of Mushrooms for Food and Agriculture (Anon. 1998) was to start a programme to document, collect and conserve wild edible and useful mushroom germplasm in developing countries, especially in Africa, tapping indigenous knowledge as much as possible. The current studies on domestication of Tanzanian edible mushrooms being undertaken by the Applied Microbiology Unit of the University of Dar es Salaam, under a Sida-SAREC Project, is an important step in line with the above recommendation.

One of the major advances in the past few decades which has radically transformed the field of systematics and taxonomy is the application of molecular genetic techniques to species identification. DNA typing relies on standard molecular methodologies such as PCR DNA amplification and Restriction Fragment Length Polymorphism (RFLP) mapping that may be easily applied to the taxonomic classification of any living system. Since molecular analysis compares stable molecular attributes instead of potentially

variable morphological traits such as environmentally influenced traits (Alexopoulos *et al.* 1996), the problems inherent in classical taxonomy can be avoided. The strength of this approach has led to its wide spread acceptance as the method of choice for species determination among animals, plants, and microorganisms. Molecular techniques also reflect evolutionary relationships between organisms, replacing the old practical systems based on morphology of dubious evolutionary information (Hibbett *et al.* 1997). The modern systems are important when screening for characters of industrial importance among evolutionary related organisms.

Classical mushroom taxonomy is not an exact science due to gaps and inconsistencies in morphology-based identification. Polymerase Chain Reaction (PCR) in combination with RFLP analysis provides an efficient means of typing a variety of fungal isolates (Bruns *et al.* 1990, 1991, Gardes *et al.* 1991, Foster *et al.* 1993, Henrion *et al.* 1994). The application of molecular identification techniques to mushroom taxonomy has the potential of facilitating the characterisation of relationships between fungal species of commercial importance.

The PCR technique allows multiplication, or amplification, of target DNA sequences from a sample containing as little as a single target DNA molecule. In PCR, a specific region of DNA, a single gene for instance, is selectively amplified by multiple rounds of *in vitro* replication by a specialized DNA polymerase called *Taq* polymerase. A pair of DNA oligonucleotides act as replication primers and delimit the region of the target molecule that will be amplified (Brown 1995). PCR products are linear DNA fragments which can be separated by gel electrophoresis and visualized as bands on ethidium bromide-stained gels. Since PCR requires the specific hybridization of selected primers, the "border" sequences of the amplified region must be known. Thus, in primer targeted PCR, the amplified products represent well-characterized regions. For instance, in mushrooms, a common target for molecular analysis is the internal transcribed region of ribosomal DNA (ITS of rDNA). While rDNA gene sequences are highly conserved, the intervening ITS regions are highly polymorphic and thus provide sequence variability which enables distinction among different strains and species. Thus, the rDNA ITS region is a useful molecular marker for taxonomic and phylogenetic studies.

The sequence of amplified PCR products can be compared by first determining the nucleotide sequence directly by standard sequencing methods, but this approach is time consuming and requires a sufficient amount of starting material. A simpler approach is RFLP mapping, in which the number and location of restriction sites is determined for each PCR-amplified product. RFLP mapping can be performed quickly and requires only a small amount of sample material. Restriction endonucleases are enzymes which cleave DNA at specific nucleotide recognition sequences,

called restriction sites. The fragments that result from restriction endonuclease digestion of a DNA molecule are called restriction fragments, and may be easily resolved by gel electrophoresis. Restriction fragment sizes are stable genetic attributes, and will segregate among progeny in the same manner as the alleles of a gene. Thus, RFLP patterns can be used as "molecular fingerprints" to distinguish individual or genotypes within or among species. RFLP analysis of rDNA ITS regions has often been used to distinguish among fungal strains and species. For example, seven species of *Sclerotina*, six species of *Candida*, and 15 different intraspecific groups of *Rhizoctonia solani* were distinguished by rDNA RFLPs (Kohn *et al.* 1988, Magee *et al.* 1987). In some cases the amount of intraspecific variations was found to be considerably higher than expected suggesting artifacts caused by broadly defined morphological species that could include several distinct biological species. However, intra species variation in rDNA ITS between strains of great geographical or temporal distances must be considered (Johannesson *et al.* 1999).

The present paper reports on the use of RFLP analysis of PCR products to ascertain the identity of mycelia isolated from fruiting bodies of some Tanzanian edible mushrooms collected from the wild.

METHODS

Sample collection

Nine mushroom morpho-species collected from Dar es Salaam, Ubenazomozi in Bagamoyo and Amani (Tanga) in April 1998 were used in this study. The selected strains are all saprophytic and hence have a potential for cultivation. These strains are part of a larger collection aiming at building up a national data base. Preliminary morphological identification was done in the field with the assistance of mushroom taxonomist, Dr. B. Buyck from Paris Museum of Natural History. Mycelia were cultured on nutrient/malt extract agar from freshly obtained tissue. Small (about 3x4 mm) pieces of fresh samples for DNA extraction were preserved in Cetyltrimethylammoniumbromide (CTAB) buffer. The remaining fruiting bodies were dried using mushroom dryers at 50 °C for 3 - 8 hours. The following mushroom morpho-species were collected:

1.	*Oudemansiella* sp.	code 98.086	Mycelia and dried mushroom
2.	*Coprinus cinereus*	code 98.091	dried and CTAB-preserved mushroom
3.	*Coprinus* sp	code 98.136	mycelia and dry mushroom
4.	*Pleurotus sajor-caju*	code 98.129	mycelia and dry mushroom
5.	*Pleurotus sajor-caju*	code 98.131	mycelia and dry mushroom
6.	*Pleurotus sajor-caju*	code 98.132	mycelia and dry mushroom
7.	*Pleurotus* sp.	code 98.137	mycelia
8.	*Pleurotus* sp	code 98.135	dry mushroom
9.	*Pleurotus flabellatus*	Code 98.114	CTAB-preserved fresh mushroom

Fruiting body of a mushroom strain coded 98.087 which was suspected to have been interchanged with sample no. 98.086 labelled *Oudemansiella* sp. was also included for sorting purpose.

DNA extraction

DNA extraction from both dried fruiting bodies and fresh samples in CTAB buffer (preserved for over 6 months) and mycelia was done according to Henrion *et al.* (1994) with some modifications by Danell (1994). About 20 to 50 mg sample of dry or fresh (preserved in CTAB buffer) fruiting bodies or mycelia were directly crushed using plastic pestle in 1.5 ml Eppendorf tubes containing 750 µl extraction buffer (5 M NaCl, 1 M Tris-HCl pH 9.0, 0.5 M EDTA, 10% CTAB, 0.2% -Mercaptoethanol) until the mixture was completely homogenized. The tubes were then incubated at 65 °C for 60-90 minutes. After centrifugation for 5 minutes at a maximum speed (14,000 rpm in a micro-centrifuge), an equal volume of chloroform was added to the supernatant and vortexed for 10 seconds followed by 10 minutes centrifugation at maximum speed. To precipitate DNA, 2 volumes of pre-chilled iso-propanol were added to the supernatant, mixed gently and incubated at -20 °C for 2 hours. The DNA was pelleted by centrifugation for 20 minutes at maximum speed and washed twice with 300 µl of ice cold 70% ethanol. After drying for 1 hour at 60 °C (or overnight at room temperature), the DNA pellet was dissolved in 50 µl of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) and stored at 4 °C until use.

PCR

The DNA isolated from fruiting bodies and mycelia was diluted 1:100 - 1:1000 using sterile milli-Q water. The rDNA ITS region was then amplified using ITS1 and ITS4 primers. These primers have been observed to amplify the entire ITS region plus a small portion (approximately 58 bp) of the 18S and 25S rRNA genes from the DNA of various fungi (Gardes *et al.* 1991). A volume of 25 µl of diluted DNA (template DNA) was mixed with an equal amount of PCR mixture containing 5.0 µl of 10x PCR buffer, 2.5 µl of 50 mM $MgCl_2$, 2.5 µl of 10 mM dNTP, 1 µl of primer ITS1 (sequence: TCCGTAGGTGAACCTGCGG), 1 µl of primer ITS4 (sequence: TCCTCCGCTTATTGATATGC), 0.25 µl of 5 units/(ml Taq polymerase, and 15.25 (ml of sterile milli-Q water. Controls with no DNA were done in every series of amplification to test for the presence of contamination of reagents and reaction buffers. PCR (35 cycles) was performed in a PE System 24000 thermocycler fitted with a heated lid using the following temperature profile:

Denaturation -	1)	95 °C for 3 minutes (initial separation of DNA strands)
	2)	95 °C for 1 minute 18 seconds (cycle begins: separation of DNA strands)
Primer annealing -	3)	48 °C for 22 seconds (primers anneal to DNA)
Primer extension -	4)	72 °C for 1 minute 10 seconds (synthesis of DNA, then back to 2)
	5)	72 °C for 10 minutes (final synthesis of DNA)
	6)	Reactions were stopped by chilling to 4 °C.

Gel Electrophoresis

The purity and length of the amplified DNA was examined by gel electrophoresis on 1 % agarose gel prepared in 1x Tris-Borate EDTA (TBE) buffer. Loading was done with 12 µl PCR product mixed with 2 µl loading buffer (10x): 0.25% bromophenol-blue plus 50% glycerol in 1x TBE buffer. A size marker lane containing 4 µl of 100 bp ladder (Boehrnger Mannheim) was included in every gel. "Minigels" (7x10 cm) were run for about 45 minutes at a low voltage range of 80-90 V. Higher voltage range of 140-150 V was used for bigger gels (10x15 cm). DNA was stained in ethidium bromide (10 mg/l) for 40 minutes, and destained in water for at least 30 minutes. Band patterns were visualised on a UV transilluminator, and photographed using a polaroid camera and/or scanner.

RFLP analysis

Restriction digestion was carried out on the PCR products of mycelial DNA to compare it with RFLP of fruiting bodies from which they were isolated. 10 µl of amplified DNA was digested by restriction enzymes (2 units) *Mbo I* (5'-∧GATC-3'), *Hinf I* (5'G∧ANTC-3'), and *Hae III* (5'-GG∧CC-3') for 3 hours at 37 °C. These enzymes have been found to have restriction sites in the ITS region of many fungi (Gardes *et al.* 1991, Henrion *et al.* 1992) and thus, the RFLP patterns observed allow distinction between fungal species and strains. The fragments were separated by electrophoresis on 1.5-2 % high quality agarose ran for 3 hours at 93 V.

RESULTS AND DISCUSSION

The results of an initial experiment to confirm if mycelia belonged to the fruiting body from which they were isolated are presented in Figure 1.

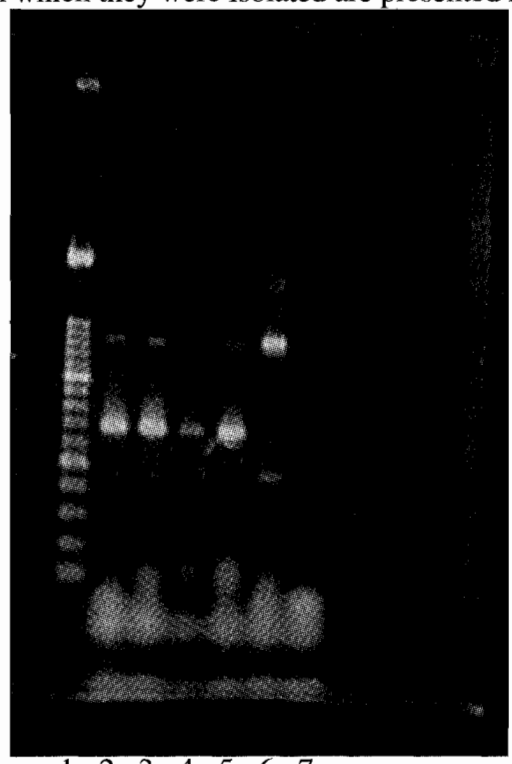


Fig. 1: Gel electrophoresis showing amplification of fungal rDNA ITS of mycelia and fruiting body. Lane 1: 100 bp ladder marker; Lane 2: DNA from *Coprinus* sp (98.136) mycelia diluted x250 before amplification; Lane 3: DNA from *Coprinus* sp mycelia diluted x500; Lane 4: DNA from *Coprinus* sp fruiting body diluted x100; Lane 5: DNA from *Coprinus* sp fruiting body diluted x250; Lane 6: Positive control - DNA from *Cantharellus;* Lane 7: Negative control (without DNA)

The ITS region of both mycelia and fruiting body of *Coprinus* sp was successfully amplified at all DNA dilutions used. A weak band was observed in lane 4 because some DNA was accidentally spilled during loading of the gel. The gel electrophoresis results gave identical banding pattern and fragment sizes (700 bp) of DNA from mycelium and its corresponding fruiting body suggesting the correct identity of the isolated mycellia. A remarkable difference in the band size between *Coprinus* DNA and *Cantharellus* DNA (control) was observed. No banding was seen in the negative control.

The results of the RFLP analysis of amplified ITS DNA are presented in Figure 2.

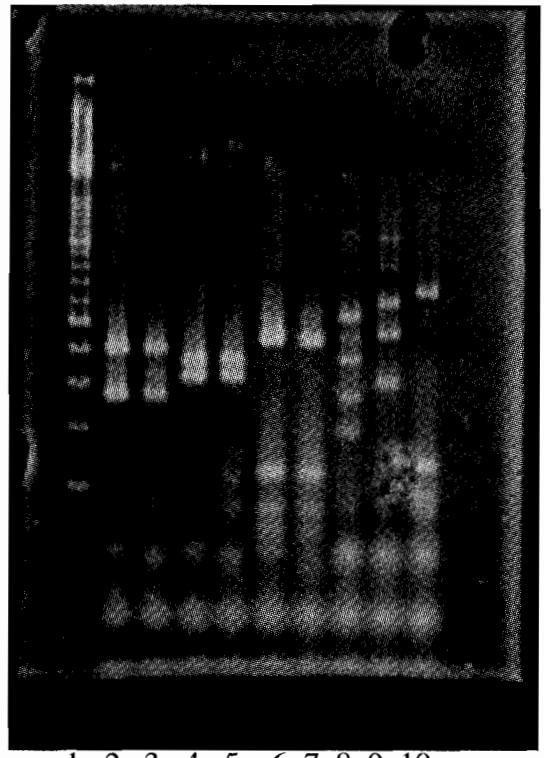


Fig. 2: RFLP analysis of the amplified ITS of mycelia and fruiting body DNA of *Coprinus* sp. Lane 1: 100 bp ladder marker; Lane 2: Mbo I-digested mycelia DNA; Lane 3: Mbo I-digested fruiting body DNA; Lane 4: Hinf I-digested mycelia DNA; Lane 5: Hinf I-digested fruiting body DNA; Lane 6: Hae III-digested mycelia DNA; Lane 7: Hae III-digested fruiting body DNA; Lanes 8 9 and 10 are MboI, HinfI and HaeIII – digested *Cantharellus* (control) DNA, respectively

Each of the three restriction enzymes produced a unique restriction pattern and fragment sizes on the same DNA material, clearly showing differences in the

restriction sites on the amplified DNA. However, identical restriction pattern was observed for both mycelia and fruiting body DNA by the three enzymes used. These results confirmed that the DNA from mycelia and fruiting body were identical.

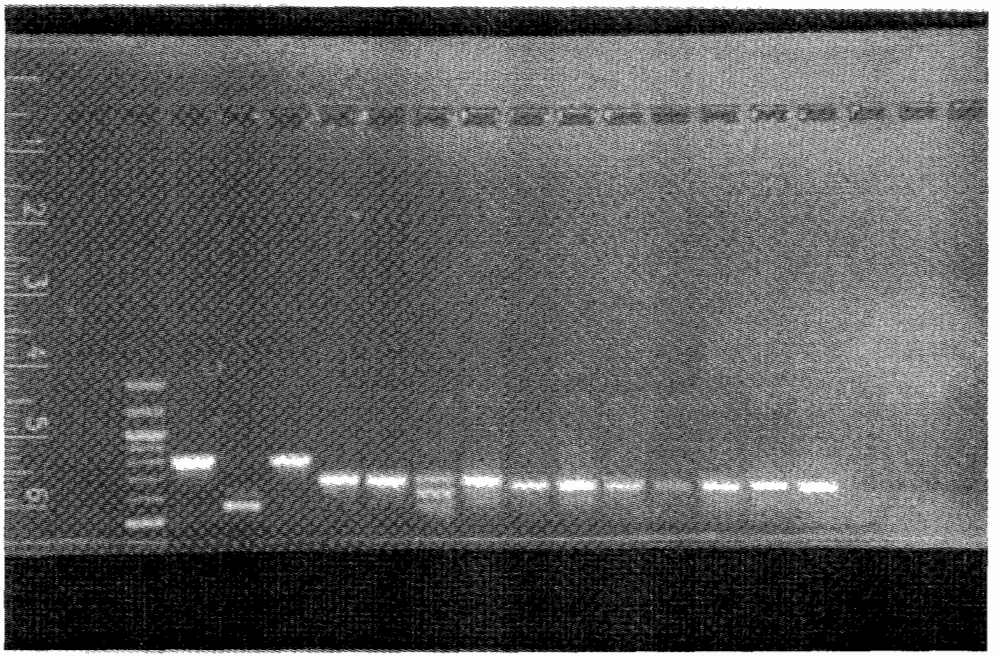
PCR amplification of the rDNA ITS region and RFLP analysis were carried out on 14 mushroom samples of the genera *Oudemansiella, Coprinus* and *Pleurotus*. Figure 3 shows the amplified ITS products of the 14 mushroom samples studied. The length of the amplified DNA varied from 600-800 bp. Similar length sizes have been recorded in 10 species of ectomycorrhizal fungi (Gardes *et al.* 1991). A remarkable difference in the banding size was observed between mycelia DNA (lane 2, with 800 bp) and its corresponding fruiting body DNA (lane 3, with 600 bp) of mushroom specimen 98.086 identified as *Oudemansiella* sp. However, the banding pattern of mycelia DNA matched very well the fruiting body DNA (lane 4) of specimen 98.087. This confirmed our earlier suspicion that there was a mislabelling of specimen, interchanging the *Oudemansiella* fruiting bodies with another mushroom specimen (*Russula* sp.). Thus, we concluded that mushroom specimen 98.087 was *Oudemansiella*.

The majority of amplified products of *Coprinus* and *Pleurotus* species were of approximately 700 bp. A clean amplification product was obtained in the CTAB-preserved mushroom as compared to a dried sample of *Coprinus cinereus* 98.091 (see Fig. 3). Since both fresh CTAB-preserved and dried samples had an identical band. The extra bands observed in the dried specimen could have arisen from contamination by other fungi or bacteria. Mushroom samples of *Pleurotus flabellatus* 98.114 preserved in CTAB buffer also yielded a good PCR product. Although the CTAB buffer we used did not contain saturated NaCl, good DNA material was obtained in samples stored on it for over 6 months at ambient temperatures. Saturated NaCl-CTAB solution has been reported as a better method of preservation of leaves for DNA extraction than the herbarium style of drying (Rogstad 1992). Our use of CTAB solution without saturated NaCl (reported in this paper) could have several advantages. The method can be utilized even at remote sites, it requires tiny amounts of mushroom tissue, and it saves time and dispenses with mains power when compared to drying or freezing. However, the effectiveness of CTAB (without saturated NaCl) for preservation of fungal specimens needs to be tested on several fungal taxa.

RFLP analysis of amplified rDNA ITS

Figures 4 and 5 depict the variability of the RFLP fragments among the mushroom species, digested with Hinf I and Mbo I respectively (Hae III results are not shown). All mushrooms studied had restriction sites for all 3 enzymes used. In all cases, the banding pattern and fragment sizes of mycelia resembled those of the corresponding fruiting bodies from which they were isolated. The identity of species was confirmed by comparing the banding

patterns with the known specimens used as references. These results provided additional evidence that the *Oudemansiella* mycelia (98.086) and the fruiting body (98.087) which carried a label of another species, were identical.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig.3. Gel electrophoresis showing amplification by PCR of the rDNA ITS in mycelia and fruiting body of 14 mushroom specimens. Lane 1: 100 bp ladder; Lane 2: mycelia of *Oudemansiella* sp. 98.086; Lane 3: fruiting body of *Oudemansiella* sp. 98.086; Lane 4: fruiting body of specimen *Russula* sp.98.087 suspected to be *Oudemansiella* sp; Lane 5: mycelia of *Coprinus* sp 98.136; Lane 6: fruiting body of *Coprinus* sp. 98.136; Lane 7: fruiting body of *Coprinus cinereus* 98.091 (dried specimen); Lane 8: fruiting body of *Coprinus cinereus* 98.091 (fresh in CTAB); Lane 9: mycelia of *Pleurotussajor-caju* 98.129; Lane 10: mycelia of *Pleurotus sajor-caju* 98.131; Lane 11: mycelia of *Pleurotus sajor-caju* 98.132; Lane 12: fruiting body of *Pleurotus sajor-caju* 98.132 (reference); Lane 13: mycelia of *Pleurotus* sp. 98.137; Lane 14: fruiting body of *Pleurotus* sp 98.135; Lane 15: fruiting body of *Pleurotus flabellatus* 98.114 (reference), Lane 16: control- without DNA

Restriction enzymes *Hinf I* (Fig. 4) and *Hae III* (data not shown) categorized the mushrooms analyzed into four distinct groups. The first group contained *Oudemansiella* sp. (lanes 2-3), the second contained *Coprinus cinereus* (lanes 4-6), the third contained *Pleurotussajor-caju* (lanes 7-11), and the fourth group contained *Pleurotusflabellatus* (lanes 12-13). However the two lower

bands in lane 10 are faintly visible in this figure, though they were clearly visible on the gel. *Mbo I* produced similar groups to those of *Hinf I* and Hae III except for the *Coprinus* sp (Fig. 5). Similarly, the lower fragment on lane 10 is not clearly visible in this Figure.

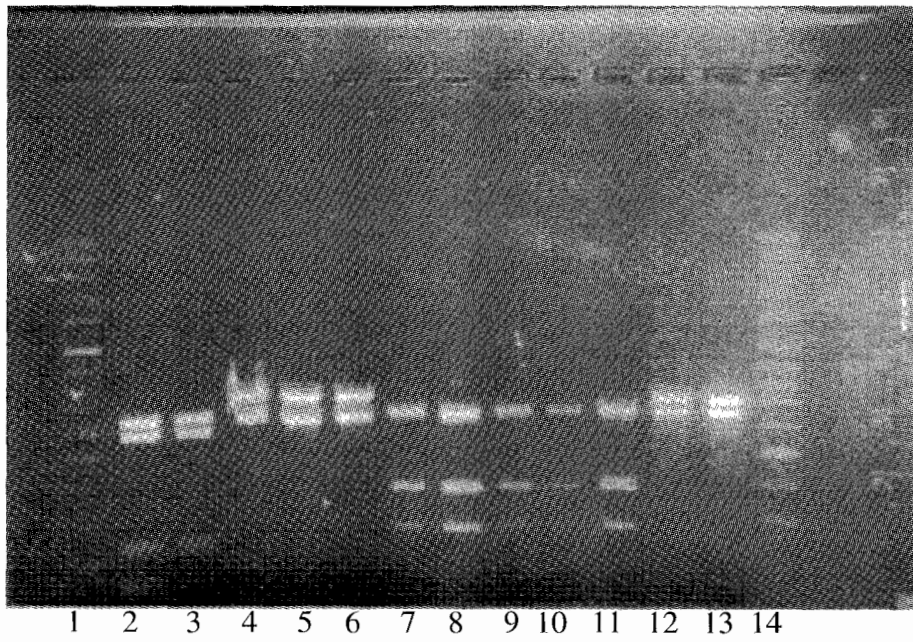


Fig. 4: RFLP analysis of amplified ITS using restriction enzyme *Hinf I*. Lane 1: 100 bp ladder; Lane 2: mycelia of *Oudemansiella* sp. 98.086; Lane 3, fruiting body of *Oudemansiella* sp. 98.087; Lane 4: mycelia of *Coprinus* sp 98.136; Lane 5: fruiting body of *Coprinus* sp. 98.136 ; Lane 6: fruiting body of *Coprinus cinereus* 98.091(fresh in CTAB); Lane 7: mycelia of *Pleurotus sajor-caju* 98.129; Lane 8: mycelia of *Pleurotussajor-caju* 98.131; Lane 9: mycelia of *Pleurotus sajor-caju* 98.132; Lane 10: fruiting body of *Pleurotus sajor-caju* 98.132 (reference); Lane11: mycelia of *Pleurotus* sp. 98.137; Lane 12: fruiting body of *Pleurotus* sp 98.135; Lane 13: fruiting body of *Pleurotus flabellatus* 98.114 (reference), Lane 14: 100 bp ladder.

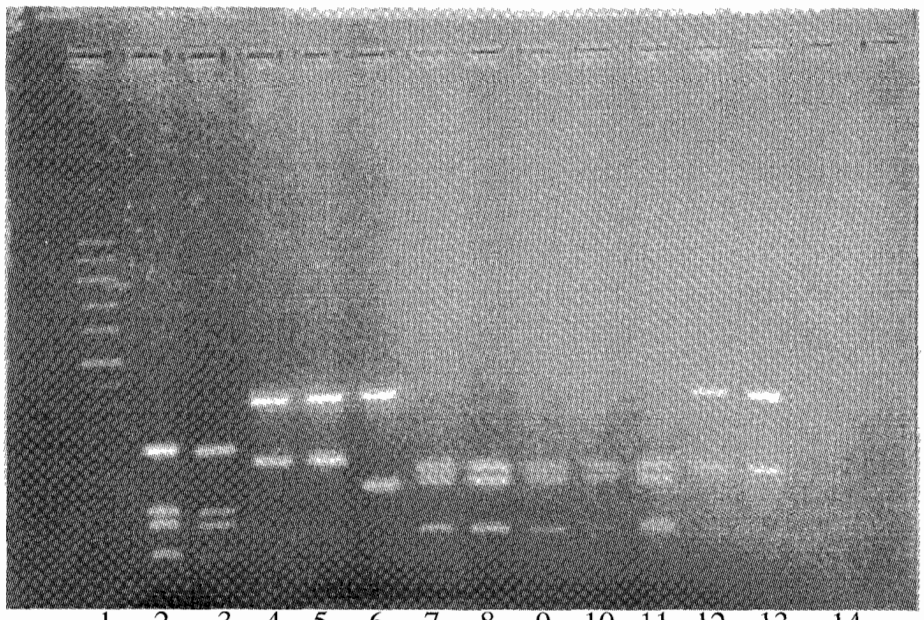


Fig. 5: RFLP analysis of amplified ITS using restriction enzyme *Mbo I*. Lane 1: 100 bp ladder; Lane 2: mycelia of *Oudemansiella* sp. 98.086; Lane 3, fruiting body of *Oudemansiella* sp. 98.087; Lane 4: mycelia of *Coprinus* sp. 98.136; Lane 5: fruiting body of *Coprinus* sp. 98.136 ; Lane 6: fruiting body of *Coprinus cinereus* 98.091 (fresh in CTAB); Lane 7: mycelia of *Pleurotus sajor-caju* 98.129; Lane 8: mycelia of *Pleurotus sajor-caju* 98.131; Lane 9: mycelia of *Pleurotus sajor-caju* 98.132; Lane 10: fruiting body of *Pleurotus sajor-caju* 98.132 (reference); Lane 11: mycelia of *Pleurotus* sp. 98.137; Lane 12: fruiting body of *Pleurotus* sp 98.135; Lane 13: fruiting body of *Pleurotus flabellatus* 98.114 (reference), Lane 14: control- without DNA

The fragment sizes of *Coprinus* sp 98.136 from Dar es Salaam were different from those of *Coprinus* 98.091 from Ubenazomozi, Bagamoyo. The results indicate that *Mbo I* restriction sites in the two mushrooms were different, though phenotypically the two mushrooms were identified as one species, *Coprinus cinereus*. This observation is supported by RFLP results using *Hinf I* and *Hae III*. Direct ITS sequencing will be required in this case, in order to correctly categorize the samples as related strains or distinct species. Restriction fragment length polymorphism of the amplified ITS has been reported among different geographical fungal isolates within a species (Henrion *et al.* 1992). The two localities from which the mushrooms were collected are separated by 100 km and differ in altitude and proximity to the sea. No such length polymorhism was shown in *Pleurotus* species 98.137 collected from high altitudes, Amani, Tanga, and those collected from Dar e Salaam.

While conventional methods for correlating mycelia with fruiting bodies are difficult and sometimes impossible to achieve, PCR amplification and RFLP analysis can be carried out in a matter of hours. The ongoing Sida-SAREC project on the characterization and domestication of wild mushrooms will incorporate this method to trace the identity of fruiting bodies to suspected source mycelia. Actually we now dare to continue working with the newly isolated *Oudemansiella* strain for cultivation.

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